DOES IT HURT TO BE CLEAN? THE ROLE OF DISINFECTANTS IN ANTIBIOTIC RESISTANCE GENE AQCUISITION

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ABSTRACT

Nosocomial, or hospital-acquired infections, are a common occurrence. As nosocomial infections are increasingly resistant to multiple antibiotics, first-line treatments often fail. Despite efforts to decontaminate surfaces and high-touch areas in hospitals, inanimate surfaces can still harbor bacteria for hours to months, providing a potential reservoir of multi-drug resistant (MDR) pathogens. Studies have shown that low levels of antibiotics and two common biocides, triclosan and chlorhexidine, can promote horizontal gene transfer. Cleaning hospital surfaces is done to prevent the spread of bacteria, but I hypothesize that this behavior may promote the acquisition of antibiotic resistance genes. To test this hypothesis, I first characterize the bacterial communities contaminating hospital surfaces and the potential for transformation of contaminating DNA. I then evaluate the effects of disinfectants on specific bacteria. Finally, I assess the potential of disinfectant induced horizontal transfer of antibiotic resistance genes. I hope to better understand how antibiotic resistance genes can be transferred in surface communities in response to disinfectant treatment, and ultimately draw conclusions to inform best hospital cleaning practices.

BIOGEOGRAPHICAL SKETCH

Cara Pardon graduated from the University of Wisconsin-La Crosse in May 2015 with a degree in Cellular and Molecular Biology, with a Chemistry minor. She entered Cornell's Genetics, Genomics, and Development program in August 2015, graduating with a Master of Science in Genetics, with a Microbiology minor, in August 2018.

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CHAPTER 1

INTRODUCTION

The CDC estimates that one in every 25 patients in a healthcare environment has one or more nosocomial infections. Moreover, 1/9 of these patients in acute care hospitals, are likely to die[1]. The most common infections include: pneumonia, gastrointestinal tract illnesses, urinary tract infections, bloodstream infections, and surgical site infections[2]. Top causative pathogens include *Clostridium difficile*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Escherichia coli*, *Enterococcus* species, *Pseudomonas aeruginosa*, *Streptococcus* species, *Enterobacter* species, and *Acinetobacter baumannii*[2]. Interestingly, many related strains of bacteria are often found as commensals, not pathogens, such as *Enterococcus* species and *E. coli* in the gut.

Unfortunately, hospital-acquired infections are increasingly resistant to multiple antibiotics. The rate of resistant bacteria to multiple classes of antibiotics shows an increasing trend over the past fifteen years[3]. In 2017, a woman in Nevada died from a *K. pneumoniae* infection resistant to all 26 antibiotics used in the U.S.[4] Antibiotics provide direct selection for resistance-conferring mutations in a population, allowing resistant bacteria to propagate. Adaptive resistance to antibiotics is also observed[5]. Do disinfectants also select for MDR pathogens?

The potential to acquire an MDR infection from a hospital often depends on environmental contamination: A doctor did not properly wash their hands in between patients; a highly contagious patient was not isolated; or surfaces became contaminated with bacteria from routine touching. Bacteria present on high-touch surfaces, such as hospital bed rails, can persist for long periods of time. Kramer *et al.* found that most gram-positive bacteria can survive for months on dry surfaces, including clinically relevant *Staphylococcus aureus* and *Enterococcus* species[6]. Gram-negative

bacteria were found to persist longer than gram-positive bacteria, again up to many months, including *E. coli*, *Acinetobacter* species, and *Pseudomonas aeruginosa*[6].

Hospitals try to eliminate long-lasting surface pathogens with cleaning protocols. Biocides (bacterial inactivating chemicals) are used as antiseptics and disinfectants[7]. According to the CDC, there are multiple reasons to use disinfectants on hospital surfaces, including: 1. Hospital floors become contaminated with bacteria from the air and through contact with shoes, spills, etc.; 2. With use of only a detergent, the detergent itself becomes increasingly contaminated and can inadvertently spread bacteria; 3. Equipment contaminated with blood or body fluids, as well as the surrounding noncritical surfaces like bedrails need to be decontaminated; and 4. Critical surfaces contaminated with blood or other potentially infectious substances are required by OSHA to be decontaminated[9].

Although their mechanisms vary, disinfectants are antimicrobial products that are used on inanimate surfaces. I will focus on three commonly used hospital disinfectants: Hydrogen peroxide (H_2O_2), bleach (NaOCl), and glutaraldehyde ($C_5H_8O_2$). H_2O_2, used on high-touch surfaces and medical fabrics and equipment, has broad-spectrum activity against bacteria, yeast, fungi, viruses, and spores[7]. It is an oxidizing compound, producing free radicals through the Fenton reaction[8]. Iron and hydrogen peroxide react to form hydroxyl radicals:

$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^- + OH^{\bullet}$ (1)
$OH^{\bullet} + H_2O_2 \rightarrow HO_2^{\bullet} + H_2O$ (2)
$Fe^{3+} + {}^{\bullet}HO_2 \rightarrow Fe^{2+} + H^+ + O_2$ (3)
$Fe^{2+} + {}^{\bullet}HO_2 \rightarrow Fe^{3+} + HO_2^-$ (4)
$Fe^{2+} + OH^{\bullet} \rightarrow Fe^{3+} + OH^-$ (5)

Reactive oxygen species increase cell reactivity and damage DNA, proteins, lipids, and bacterial iron-sulfur clusters. H_2O_2 is also used in combination with other disinfectants such as peracetic acid[9]. A concentration of 0.6% hydrogen peroxide has been shown to inactivate nosocomial

pathogens in urine, including *S. aureus*, *E. coli*, and *Pseudomonas* species, with longer contact time needed for organisms with high catalase activity like *S. aureus*[9].

Bleach is also a common oxidizing agent used to clean a variety of hospital surfaces, blood spills, and contaminated needles[9]. Releasing hypochlorite ions in solution, sulfhydryl groups are irreversibly oxidized, preventing proper enzymatic functions[7]. Bleach's high pH can also affect membrane integrity[10]. Cells may lose their intracellular contents, protein synthesis can be inhibited, and breaks in the DNA through oxidation can occur. Regular bleach can inactivate 10^6 *C. difficile* spores in less than ten minutes at a concentration of 5,000 ppm chlorine. 100 ppm of chlorine has been shown to kill 10^6-10^7 *S. aureus* and *P. aeruginosa*[9]. (A 0.6% solution of bleach contains around 6000 ppm of chlorine.)

Lastly, glutaraldehyde is a common hospital disinfectant used on surgical and other procedural equipment, such as endoscopes and dialyzers. An alkylating agent interacting strongly with bacterial cell walls and membranes, glutaraldehyde cross-links proteins in the outer layers of bacteria and alkylates sulfhydryl, hydroxyl, carboxyl, and amino groups within the cell[9]. Glutaraldehyde is effective against bacteria and spores, as well as fungi and viruses. Glutaraldehyde can be made sporicidal at pH 7.5-8.5. *M. tuberculosis* at a concentration of 10^4-$10^{6.4}$ could be inactivated with 20 minutes of 2% glutaraldehyde exposure. Unfortunately, although multiple studies have shown that common hospital antibiotic resistant bacteria, like *P. aeruginosa* and *Enterococcus* species, do not exhibit decreased susceptibility to disinfectants, even with cleaning protocols in place, the spread of resistant pathogens is still common[9].

Bacterial resistance to antibiotics and disinfectants can be due to a number of innate or acquired mechanisms. Impermeability is a first line of protection, specifically for gram-negative bacteria which have a protective outer membrane in addition to their cell wall and inner membrane.

This limits uptake of a disinfectant. Bacteria can also enzymatically destroy or alter the compound. Examples include: catalases, superoxide dismutase, and glutathione peroxidase, which reduce hydrogen peroxide, and β-lactamases, which hydrolyze β-lactam ring molecules. Resistance to chlorine and H_2O_2 has been attained through overexpression of catalases or through induction of the *oxy*R or *sox*RS systems in *E. coli*[11]. Efflux pumps are a common mechanism for biocide resistance, associated with resistance to tetracyclines, quaternary ammonium compounds, and heavy metals[12]. It is my understanding that little else is known about the resistance mechanisms to H_2O_2, bleach, or glutaraldehyde.

Resistance to multiple biocides or antibiotics can be due to cross- or co-resistance. Cross-resistance can occur when two substances target the same gene or pathway. Resistance to one substance increases resistance to the other. Co-resistance occurs when resistance genes are located together on a mobile element[13]. Quaternary ammonium compounds (QACs) are widely studied disinfectants exhibiting co-resistance. The *qac* gene efflux pumps have been found on plasmids carrying β-lactamases, integrons, and aminoglycosides, and are responsible for exporting QACs and other compounds, such as Ethidium Bromide[13]. Braoudaki and Hilton showed that cross-resistance to antimicrobial compounds is common. *E. coli 0157* strains acquired resistance to triclosan after only two sub-MIC (minimum inhibitory concentration) treatments[5]. The adapted *E. coli* strain was also much less susceptible to antimicrobials including chloramphenicol, tetracycline, imipenem, trimethoprim, erythromycin, and other biocides including chlorhexidine[5]. In another example, Pal *et al.* quantified the co-occurrence of antibiotic resistance genes (ARGs), biocides and metal resistance genes (BMRGs) using publicly available, fully sequenced bacterial genomes and plasmids[14]. Analysis showed that mercury resistance genes co-occurred on plasmids carrying the *qacEΔ1* gene for quaternary ammonium compounds. Importantly, several clinically

relevant taxa often carried both ARGs and BMRGs. BMRGs were found in 86% of bacterial genomes, co-occurring with ARGs in 17% of these cases. Also, conjugative plasmids were more likely to carry ARGS and BMRGs[14]. These conjugative plasmids can be transferred horizontally.

Most bacteria acquire resistance genes through horizontal gene transfer. Horizontal gene transfer (HGT) occurs through three mechanisms: transformation, transduction, and conjugation. Transformation is the direct uptake of DNA from the environment. Transduction involves the transfer of DNA through a bacteriophage infection. Lastly, conjugation allows the transfer of genes directly from one neighboring bacteria to another. Some bacteria are naturally competent and there are three evolutionary hypotheses as to what beneficial role competence plays. The first hypothesis is one of genetic diversity. Bacteria have the ability to pick up environmental DNA in the hopes of beneficially diversifying their genome. A second hypothesis is that bacteria take up DNA to acquire nucleotides as a food source. Lastly, natural competence acts as a DNA damage repair hypothesis. Bacteria take up DNA that could provide a template for DNA repair. This last hypothesis seems the most likely. Competence can be induced by DNA damaging agents, which will be discussed as it relates to the bacterial response to DNA damage[15].

The SOS response in bacteria is a global, inducible system responding to DNA damage[16]. The two key proteins involved in SOS regulation are LexA and RecA. Under normal conditions, LexA prevents expression of SOS genes by binding to specific sequences in promoters of SOS genes. In the presence of DNA damage, RecA binds to single-stranded DNA and induces LexA self-cleavage[17]. Release of LexA allows for the expression of many genes, including DNA recombination enzymes and polymerases[18]. The SOS response is found in a variety of phyla, from *Gammaproteobacteria* to *Firmicutes*, though slight differences exist[19]. A notable exception in the *Firmicutes* is the pathogen *Streptococcus pneumoniae*[18].

Antibiotic-induced SOS response increases HGT. Beaber *et al.* observed that ciprofloxacin induced transfer of a 100-kb, resistance-conferring integrating conjugative element (ICE)[20]. Ubeda *et al.* concluded that SOS-induced with fluoroquinolones promoted the spread of *S. aureus* pathogenicity islands[21]. Sub-MIC levels of gentamicin, ciprofloxacin, and cefotaxime were shown to induce ROS formation and multi-drug resistance in a methicillin-resistant *Staphylococcus aureus*[22]. HGT increases are even seen in non-SOS species. Streptomycin and Kanamycin, which inhibit translation, induce competence in *S. pneumoniae*[18]. Competence can act as a substitution for the SOS response. Sub-inhibitory concentrations of fluoroquinolones also increased conjugation frequency of a plasmid in *E. coli* and induction of phage-mediated transfer in MDR *Salmonella*[23,24]. But a big question remains: Do disinfectants, specifically sub-inhibitory or low concentrations, also increase HGT?

Recent findings indicate this may indeed be the case. Jutkina *et al.* studied the effects of low concentrations of antibiotics and biocides on resistance gene transfer from sewage effluent bacteria to *E. coli*[25]. Antibiotics and two common biocides, triclosan and chlorhexidine induced HGT at low concentrations. Low levels of heavy metals have also been shown to facilitate transfer of a resistance plasmid[26]. Zhang *et al.* previously reported an increase of HGT following sub-inhibitory disinfectant treatment (H_2O_2, free chlorine, and chloramine)[27]. Is this effect seen universally across all types of bacteria, and importantly, for potential surface-dwelling nosocomial pathogens? I use multiple techniques, including sequencing, plating, and Fluorescence Activated Cell Sorting (FACS), to examine this question.

Fluorescence Activated Cell Sorting is a common technique used in a multitude of applications. Cells in a suspension are labeled with fluorescent proteins, individual cells are encased in droplets of sheath fluid, and passed through lasers/detectors set at the correct

wavelength to detect the fluorescent protein. Cells can then be separated based on their size and fluorescence. The majority of FACS applications since its invention are performed on eukaryotic cells because FACS is harder with bacterial cells. It is difficult to distinguish the small size of the microbes from cellular debris and cells cannot always be singly differentiated. A bacterial community it is also difficult to profile, as the variety of shapes, sizes, and varying reflective properties of each cell type, differ. Consequently, when performing FACS with bacterial cells, it is important to not only sort based on size, but with at least one fluorescent protein/channel.

Studying bacterial cell physiology with FACS has been widely done. Fluorescent dyes such as SYBR green, propidium iodide, and DiBAC are commonly used together to determine the proportion of viable bacterial cells in a population. SYBR green (fluoresces green) binds to DNA, and represents the total amount of DNA in a cell. Cells with a high level of green fluorescence have a high nucleic acid content and can be considered actively dividing, whereas cells with lower green fluorescence have a lower nuclei acid content and are likely not dividing. Propidium iodide fluoresces red and is membrane impermeable, therefore only able to label DNA in cells with compromised membranes. DiBAC gives information about the polarity of cells as it only enters cells which are depolarized and damaged, providing a different red fluorescence excitation from propidium iodide. Conclusions about the percentage of live/dead or viable/healthy cells can be determined based on the amount of each color of fluorescence. Other uses for bacterial FACS include elucidating host-pathogen interactions and determining bacterial response to antimicrobials or chemicals. Later, I will focus discussion on the use of FACS to study plasmid conjugative transfer.

The goal of Chapter 2, a proposal, is to understand the effects of sub-inhibitory concentrations of the disinfectants hydrogen peroxide, bleach, and glutaraldehyde, on HGT and potential MDR pathogens.

CHAPTER 2

PROPOSAL

Abstract

Nosocomial, or hospital-acquired infections, are a common occurrence. As nosocomial infections are increasingly resistant to multiple antibiotics, first-line treatments often fail. Despite efforts to decontaminate surfaces and high-touch areas in hospitals, inanimate surfaces can still harbor bacteria for hours to months, providing a potential reservoir of multi-drug resistant (MDR) pathogens. Studies have shown that low levels of antibiotics and two common biocides, triclosan and chlorhexidine, can promote horizontal gene transfer. Cleaning hospital surfaces is done to prevent the spread of bacteria, but I hypothesize that this behavior may promote the acquisition of antibiotic resistance genes. To test this hypothesis, I first characterize the bacterial communities contaminating hospital surfaces and the potential for transformation of contaminating DNA. I then evaluate the effects of disinfectants on specific bacteria. Finally, I assess the potential of disinfectant induced horizontal transfer of antibiotic resistance genes. I hope to better understand how antibiotic resistance genes can be transferred in surface communities in response to disinfectant treatment, and ultimately draw conclusions to inform best hospital cleaning practices.

Aims

1. **Characterize the microbes and DNA contaminating hospital surfaces and the transformability of genes found on these surfaces**
    A. Identify surface contaminants at Cornell Veterinary Medicine and Weill Cornell Medicine via 16s-rRNA sequencing
    B. Elucidate the different effects disinfectants have on species community
    C. Evaluate the potential for antibiotic resistance gene transfer using an *A. baylyi* model
2. **Elucidate the effects of sub-inhibitory levels of disinfectants on potential pathogens**

A. Determine the minimum inhibitory concentrations and effects of disinfectants on the viability of specific bacterial strains
B. Assess bacterial growth dynamics in the presence of disinfectants

3. **Examine the role of sub-inhibitory levels of disinfectants in horizontal gene transfer**
   A. Quantify the extent to which disinfectants increase the rate of horizontal gene transfer using competence and conjugation selective plating assays with broad host range plasmids
   B. Quantify the extent to which disinfectants increase the rate of HGT using FACS with broad host range plasmids
   C. Analyze oxidative stress through an assay for reactive oxygen species production
   D. Examine the adaptive evolution and horizontal gene transfer ability of specific species grown constantly in sub-inhibitory concentrations of disinfectants

Experimental Design and Methods

1. **Characterize the microbes and DNA contaminating hospital surfaces and the transformability of genes found on these surfaces**

Identify surface contaminants at Cornell Veterinary Medicine and Weill Cornell Medicine via 16s-rRNA sequencing

In order to directly study hospital surface bacteria and contaminating genetic material, I need access to hospitals. So, in collaboration with Weill Cornell Medicine (WCM) and Cornell Veterinary Medicine (CVM), I determined what microbes are contaminating surfaces using 16s-rRNA sequencing. Although contamination studies are widespread, this study gives insight into specific problematic contaminants of Cornell hospitals. COPAN ESwabs containing Liquid Amies were used to swab commonly contaminated surfaces in the hospitals, such as bed rails and floors

(COPAN). DNA was extracted from the swab solution using the automated Maxwell 16 Cell LEV DNA Purification Kit (Maxwell). Although the Maxwell kit has been optimized for small cell numbers, even as low as 10 cells, many samples contained very little to no DNA following extraction. Sequencing results were obtained for the majority of samples, but the amount of DNA is too little (<0.2 ng/μL) for downstream experiments. If this is the case for future swab extractions, I will use the sequencing results and literature as sources for hospital-contaminating DNA. There are many studies that look at hospital contamination[28,29,30]. In this respect, I can find common contaminating bacteria and use these as a mock community for hospital surface bacteria in future experiments.

The extracted DNA was sent to Argonne National Labs for 16s-rRNA library preparation using the Earth Microbiome Protocol and sequencing on a MiSeq platform[31]. As a control for environmental DNA which may contaminate all samples, water blanks were also library processed and sequenced. 16s-rRNA analysis will be performed using the QIIME2 pipeline[32]. Preliminary results reveal high levels of *Acinetobacter* species, up to 74% and 18% in CVM and WCM samples, respectively (Figure 1). WCM samples also contained up to 53% *Pseudomonadaceae* (Figure 1). Other contaminants include gut bacteria such as *Enterobacteriaceae* and *Bacteroides*, as well as skin or mouth bacteria like *Staphylococcus* and *Streptococcaceae* (Figure 1).

Elucidate the different effects disinfectants have on species community

To elucidate potential differences disinfectants may have on species community, I performed a PMA-seq assay[33]. PMA (Propidium Monoazide) is a cell-impermeable, DNA binding molecule. Upon excitation with strong visible light (600-watt Halogen bulb), PMA bound to DNA is excited and fluorescent. PMA prevents further amplification of DNA through steric hindrance, allowing discernment between live and dead cells. Permeable or dying cells and extracellular DNA

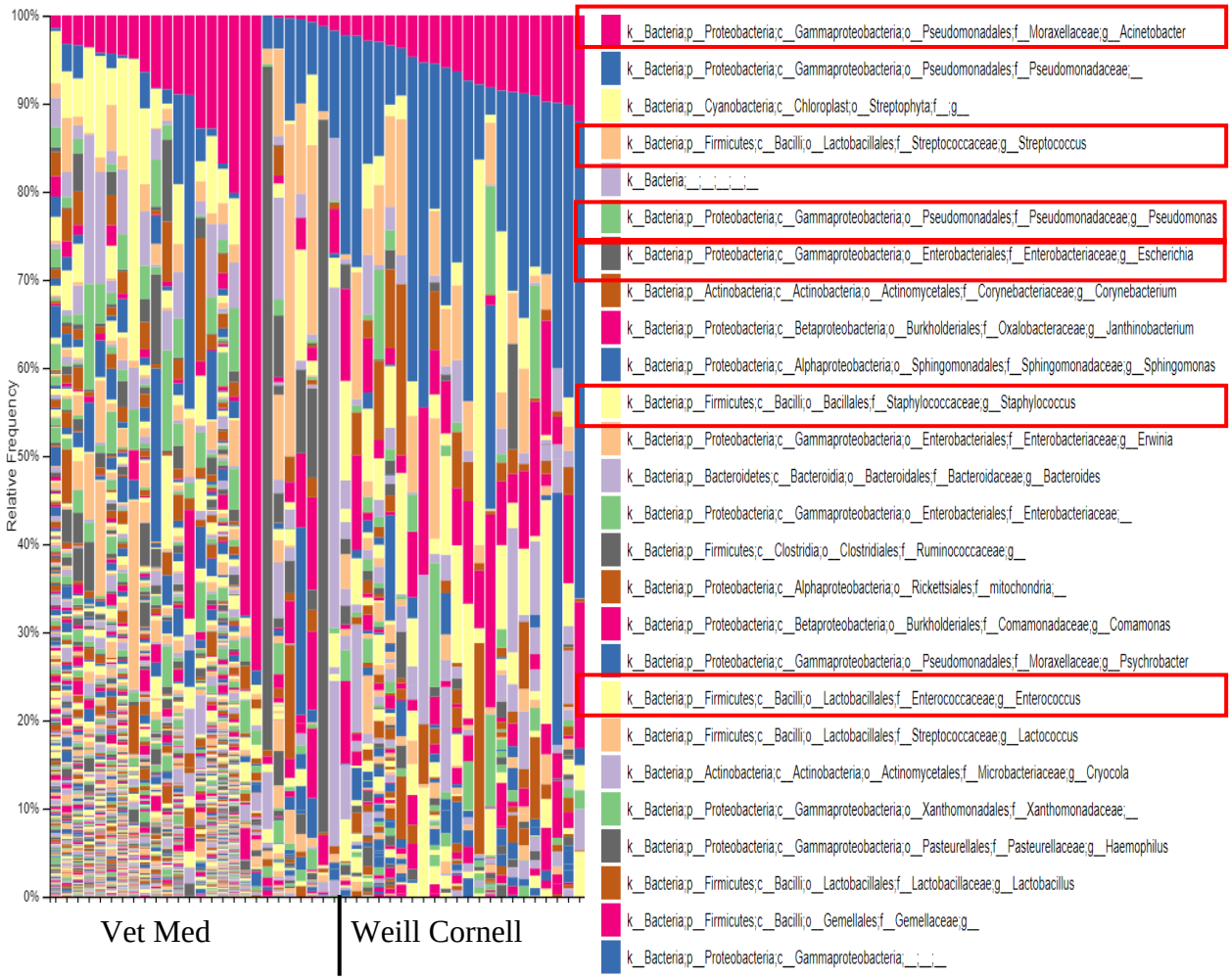

Figure 1: Representative CVM (left) and WCM surface DNA (right) at the Genus level (legend shows top 25 only). Red boxes represent genera of bacteria chosen for downstream experiments.

will be bound by PMA and will no longer be able to be amplified. Live/dead readout can be established using FACS or 16s-rRNA sequencing.

To compare the effects of disinfectants on potential surface contaminating microorganisms, I used PMA-seq to determine what percent of cells and which taxa of cells were alive or dead following disinfectant treatment. Stool from a healthy adult female was used as a model for enteric surface contaminating bacteria, as many nosocomial pathogens are found in stool. Samples were treated with varying concentrations of H_2O_2, bleach, and glutaraldehyde for two lengths of time. Because storage methods can influence microbiome composition, fresh

samples, frozen samples, and samples frozen with the addition of L-cysteine, which provides a reducing environment to remove oxygen, were also prepared and underwent PMA treatment. Results indicate no significant difference in Shannon diversity between PMA and non-PMA treated samples, indicating little to no PMA bias for specific taxa. There was also no significant differences between the various disinfectants, indicating little taxa killing bias between the disinfectants as well. From this preliminary study, PMA-seq seems to be a reliable method to detect live and dead cells within a sample. Although the efficiency of PMA can be interrupted by organic matter, PMA-seq was able to detect differences in microbe composition in stool samples. Therefore, it is likely that PMA-seq could be an efficient method to use on surface samples as well, assuming enough starting material.

Alternatively, to determine if any contaminating bacteria are viable and drug-resistant, I can directly plate hospital environmental swabs onto selective plates. Trypticase Soy Agar (TSA) and Blood Agar Plates (BAP) containing standard amounts of common antibiotics will be used. For example, swabs would be plated on media containing penicillin, tetracycline, vancomycin, and methicillin. Penicillin and tetracycline are very commonly used antibiotics, and resistance to vancomycin and methicillin are often associated with nosocomial infections, i.e. methicillin-resistant *staphylococcus aureus* (MRSA) and vancomycin-resistant *Enterococci* (VRE). The ability to isolate organisms directly on plates would provide important information for the Cornell hospitals on potential MDR-pathogen contamination.

Evaluate the potential for antibiotic resistance gene transfer using an A. baylyi model

What percent of the hospital surface contaminating DNA is actually transformable? What is the identity of this DNA? To answer these questions, I use an *Acinetobacter* species. *Acinetobacter baumannii* is a nosocomial gram-negative pathogen, which was susceptible to most

antibiotics in the 1970s[34]. Currently, most *A. baumannii* strains exhibit resistance to a wide range of antibiotics. *A. baumannii* contains two intrinsic β –lactamase genes conferring resistance to β-lactams, as well as efflux systems, which makes *A. baumannii* naturally less susceptible to antibiotics. Integrons and transposons are the main genetic factors associated with antibiotic resistance in *A. baumannii*[35]. Genomic analysis of *A. baumannii* strain AYE, which is endemic to France, contains an 86-kb resistance island cluster of 45 resistance genes[34]. Interestingly, Qin *et al.* found that many genes linked to drug resistance were transposon associated. Upon antibiotic treatment, transposable elements showed higher levels of expression, indicating again the possible connection between antibiotic treatment and ARG transfer[36].

It has been determined that *Acinetobacter* has a great propensity and ability to acquire genes horizontally; in other words, a high natural competence[37]. Transformation is limited by a number of factors. Species such as *H. influenza* exhibit sequence-specific uptake based on nucleotide motifs, restricting transfer with distantly related species. Plasmids and chromosomal DNA are both transformable, though plasmid transformation is more difficult. A cell needs to be able to reassemble the double-stranded circle upon transfer. A recipient cell also needs to be able to stably integrate DNA for it to be expressed[37]. Linear plasmid transformation can be restricted by one enzyme, decreasing transformation rates up to 10,000 times. Re-circularization of linear plasmid DNA can be facilitated by a copy of the same linear plasmid, cut with a different restriction enzyme, providing a template for homologous recombination. A mixture of plasmids cut with different enzymes partially restores the transformation rate[38].

With these barriers to HGT in mind, I perform a transformation assay with *A. baylyi*, originally isolated from soil. *A. baylyi* is highly naturally transformable and safer to work with than clinically relevant *A. baumannii* because it is BSL1[39]. Natural transformation of *A. baylyi*

requires four steps. First, competence must be induced, which can be done through natural induction upon a fresh carbon source, providing an increase in nutrients. The next step is the DNA-binding step. *A. baylyi* does not exhibit sequence-specific uptake and can pick up both homologous and heterologous DNA. DNA must then be translocated across the outer and inner membranes, requiring ATP or a proton gradient. Lastly, the DNA must be recombined either through site-specific homology-independent illegitimate recombination or homology-facilitated illegitimate recombination[38]. *A. baylyi* can uptake 60 base-pairs of DNA per second *in vitro* with successful recombination of DNA fragments about 0.1% of the time[37]. Increasing fragment size from 0.3-3.8 Kb, increases transformation rates with a maximum rate of 10^{-1} transformants[38].

I incubate *A. baylyi* with DNA (~10 ng/µL) extracted from hospital surfaces in Brain Heart Infusion media, shaking at 37 C for one hour (Hardy Diagnostics, Jonathon Friedman). Controls include incubating *A. baylyi* with water or with characterized plasmids. I then perform shotgun metagenomic sequencing, using the Nextera DNA library preparation kit (Illumina). Wild-type *A. baylyi* will be paired-end sequenced, along with *A. baylyi* from the transformation experiment on an Illumina NextSeq platform. *A. baylyi* has a genome size of about 3.6 Mb. Assuming 50x coverage and 700 million reads provided by one NextSeq lane, 1,200 possible genomes or different transformants can be sequenced in one lane. The wild-type genome is assembled as a reference genome with Velvet[40]. I then perform multiple sequence alignment of the experimental *A. baylyi* to the wild-type reference with MAFFT[41].

Alternately, to avoid sequencing so many full-length genomes, most of which will be homologous, I can perform plasmid isolation for each isolate. Sequencing the plasmids of each will allow me to determine the types of plasmids able to transform *A. baylyi*. Sequencing depth can be increased if necessary, as less starting material will be sequenced. Plasmid sequences would

be assembled with plasmidSPADES and BLASTed against a database of plasmid sequences from NCBI. *A. baylyi* can also be directly plated onto TSA antibiotic plates like tetracycline and methicillin, to examine potential antibiotic resistant transformants without sequencing.

I expect much of the transformant genomes to align as the core genes of *A. baylyi*. I assume that additional gene length sequences are transformed pieces of DNA. I BLAST these resulting sequences to a number of databases. The first is to BLAST to the NCBI microbial nucleotide database to look for any microbial identifying genes, as well as the viral and fungal BLAST databases. To look for ARGs, I BLAST to the CARD database and perform an HMMER search[42,43]. As an alternative to sequencing which may be wasteful if much of the surface extracted DNA is not transformable, I can perform selective plating of *A. baylyi* to assess transformation with ARGs. If no ARGs were present in the hospital surface extracted DNA, I can still test the potential for *A. baylyi* to pick up ARGs from the environment with the addition of ARGs to the transformation assay. I expect that *A. baylyi* will be transformed with multiple fragments of DNA from a wide variety of genera, including resistance genes, though limited in fragment size as transformation of very large plasmids is difficult and rare. From this experiment, I garner information about the potential transformability of contaminating surface DNA from Cornell hospitals.

Aim 2: Elucidate the effects of sub-inhibitory levels of disinfectants on potential pathogens

Determine the MICs and effects of disinfectants on the viability of specific bacterial strains

To understand disinfectant effects on specific, clinically relevant strains of bacteria, it is important to determine minimum inhibitory concentrations (MICs) and perform viability assays (Tables 1, 2). Species chosen are common nosocomial pathogens, including genera found contaminating Cornell hospitals (Figure 1). Specific MICs for each species are determined with a

broth microdilution method[44]. Using Abcam's bacterial viability assay kit, I determine what percent of cells remain viable following disinfectant treatment.

Table 1: Species to use in the disinfectant experiments.

Bacterial Species
Enterococcus faecalis Portland
Acinetobacter baylyi ADP1
Streptococcus pneumoniae
Escherichia coli
Pseudomonas aeruginosa
Staphylococcus aureus

Assess bacterial growth dynamics in the presence of disinfectants

To understand how bacterial growth dynamics change upon disinfectant treatment, I perform growth curves. What is the rate of killing for each disinfectant and bacteria? What is the recovery growth rate for surviving bacteria? I perform these assays in a 96-well plate, measuring the optical density at 600 nm, over an 18 hour-period. Bacteria are grown in their respective growth media at 37 C in a plate reader for six hours. Then disinfectants are added (Table 2). Cells continue to be grown for another twelve hours. I expect to see differences in the recovery time of the different species depending on the disinfectant used. For example, as hydrogen peroxide is more effective against gram-positive bacteria, longer lag times for gram-positive *Staphylococcus* and *Enterococcus* compared to gram-negative are expected[7].

Aim 3: Examine the role of sub-inhibitory levels of disinfectants in horizontal gene transfer

Quantify the extent to which disinfectants increase the rate of HGT using competence and conjugation selective plating assays with broad host range plasmids

I test the hypothesis that sub-inhibitory levels of disinfectants increase horizontal gene transfer using competence and conjugation assays. For these experiments, I assay for the transfer

of broad host range plasmids from the environment and from a host population. Broad host range plasmids isolated from soil, RP4 and pKJK5, were shown to be able to invade a diverse fraction of soil bacteria, encompassing many phyla[45]. These plasmids were previously tagged with a GFP construct. Importantly, donor strains of *E. coli* were constructed carrying a *lacI*^{q} repressor upstream of the GFP gene, and a constitutively expressed chromosomally encoded red fluorescence. This means that in the donor strain, the cells will be red, but upon transfer to a new strain, the recipient will express GFP[45]. Donors and recipients can be separated based on their fluorescence. The plasmids also contain antibiotic resistance genes that allow for selective plating of transformants and transconjugants.

Table 2: Disinfectants and concentrations used/to be used.

Disinfectant	**Concentrations**
Hydrogen Peroxide	1%, 0.1%, 0.01%, 0.001%, 0.0001%
Bleach	1%, 0.1%, 0.01%, 0.001%, 0.0001%
Glutaraldehyde	1%, 0.1%, 0.01%, 0.001%, 0.0001%

For the assays, a previously established conjugation model is used[27]. Briefly, the donor *E. coli* strains are mixed with recipient cultures in a 1:1 ratio with the addition of RP4 and pKJK5 (Table 1). To minimize any influence from the media itself, bacteria are re-suspended in phosphate buffered saline (PBS) before disinfectant treatment. Bacteria are then treated with disinfectants for two minutes, washed, and outgrown in media for 12 hours. Serial dilutions are plated on plasmid-selective media containing an antibiotic that selects against the donor strain. If this method is unsuccessful, the solid-surface conjugation model used by Klumper *et al.* will be utilized[45]. If the hypothesis holds true, I expect that bacteria treated with disinfectants will exhibit higher transformation or conjugation rates than untreated cells. Although I cannot distinguish between the two, I expect the most naturally competent species, *A. baylyi* and *S. pneumoniae*, to exhibit the

largest increase in transformation following disinfectant treatment. I expect conjugation rates to increase more in the less naturally competent species, as conjugation of plasmids is more efficient than plasmid transformation. I may also be able to improve the rate of successful transformation through restriction. As mentioned previously, linearized plasmids cut with one enzyme greatly decreases transformation rates. But if I restrict the plasmids with multiple enzymes and provide homologous templates for recombination, I can potentially increase transformation and conjugation rates in my experiments.

Alternatively, species specific plasmids for each bacteria could be used, rather than the broad-host range plasmids. Through the cloning of ARGs into species specific plasmids, I can examine rates of conjugation and transformation. As the plasmids are host specific, I know that each species should have a basal transformation/conjugation rate for its plasmid. Comparing this basal rate with the rate upon disinfectant treatment would also allow me to investigate my hypothesis.

Quantify the extent to which disinfectants increase the rate of HGT using FACS with broad host range plasmids

Because the plasmids are fluorescent, in a similar manner, the experiments described above can potentially be analyzed with FACS, rather than selective plating. In these experiments, GFP readout is used as a measure of positive transformants and transconjugants. I have performed multiple FACS competence and conjugation experiments, prior to beginning the plating assays. I began with a stool sample, as many nosocomial infections are from enteric bacteria. RP4 and pKJK5, as well as host cells, were added to the sample, and then the disinfectants were added. Samples were washed after two minutes and submitted to FACS. After repeating this experiment and collecting data, it became clear that stool is too auto-fluorescent, obscuring the selection of

positive transformant and transconjugant cells. As stool also contains a large number of anaerobic bacteria, dead and dying bacteria also auto-fluoresced. Performing the experiment with stool also makes it hard to have an accurate positive control, as I do not have positive-plasmid cells for all types of stool bacteria. I moved on to specific cultures of bacteria.

Although *Vibrio cholerae* is not a very good model for nosocomial infections, I chose to use this organism for further experiments, as the lab has good positive controls for the RP4 and pKJK5 plasmids. I also began work with *Enterococcus faecalis*, though at this point in time, no positive control had been successfully transformed or electroporated with the plasmids, potentially limiting the conclusions that could be drawn from the experiments.

Upon analysis of all the samples, a few stood out as potential sources of truth to the hypothesis that disinfectant increase HGT and competence. Compared to the negative controls, both 1% bleach and 1% glutaraldehyde exhibited greater than 40% GFP+ cells, indicating large numbers of transformants or transconjugants (Figure 2). But, these results needed to be verified with microscopy and selective plating of the GFP+ cells. Following microscopy examination and plating of the plasmid-positive cells onto selective media, there was a problem. Not only was it hard to find any GFP+ cells under the scope, but nothing grew on the plates. I concluded that what I was seeing with FACS was not a real indication of HGT, but auto-fluorescence stimulated by the disinfectants themselves. The GFP+ cells that I was collecting were either not viable or not cells at all. The next step to improve the reliability of FACS would be to add a dead stain to the samples, like Propidium Iodide, to select against non-viable GFP+ cells and debris. This will likely be done in the future to move away from the conjugation and competence plating assays described earlier, as it requires fewer materials and hours to complete the experiments.

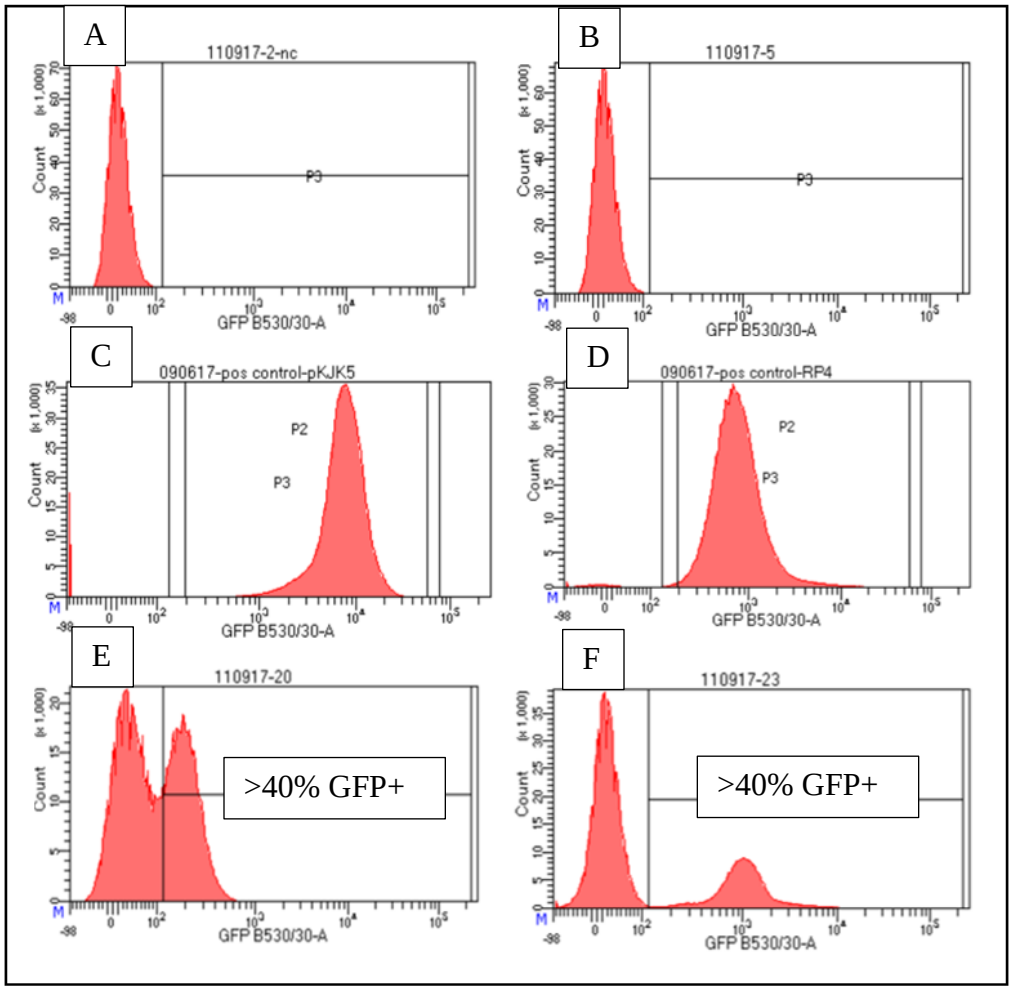

Figure 2: FACS data from competence/conjugation experiments. **A.** Negative Control, wild-type *E. faecalis* **B.** Negative Control for disinfectant *E. faecalis* **C./D.** Positive control *V. cholerae* for pKJK5 and RP4, respectively **E.** 1% Bleach *E. faecalis* **F.** 1% Glutaraldehyde *E. faecalis*

These assays highlight the importance of controls in microbial FACS analysis. Not only is it important to have negative controls (such as cells without a marker, probe, or labeled secondary antibody) and positive controls (cells that are definitely labeled) to identify and verify the correct cells to sort, it is also important to have controls of the various stages of sorting in the experiment. Cells may lyse and die (as mentioned above). It may be important to therefore, compare the unsorted with the sorted fraction or the total effluent with the original sample. FACS can place

environmental stress on cells, changing their shape and physiology during sorting. Although it was obvious upon verification methods that my results were not real, it can be difficult to verify FACS results of microbiomes. All the necessary verifications and controls should be used when analyzing microbes with FACS.

Analyze oxidative stress through an assay for ROS production

One of the most commonly used techniques for measuring redox of a cell directly is the use of 2'-7'-Dichlorodihydrofluorescin diacetate (DCF-DA)[46]. DCF-DA is cleaved by intracellular esterases into H_2DCF, which cannot diffuse out of the membrane. DCF, a fluorescent product, is produced from the oxidation of H_2DCF, allowing for measurements of fluorescence increase and accumulation of DCF in the cell[46]. DCF is not a direct measure of a specific radical or compound like H_2O_2 or $NO^{\bullet}$, rather it is an assay of general oxidative stress. Although not specific, this allows me to assay for ROS production in a variety of different disinfectant treatments, without assaying for specific radicals that may be produced in each specific condition.

Briefly, cells are treated similarly to the competence/conjugation assay, without the addition of plasmids (Tables 1, 2). Following disinfectant treatment, cells are incubated with DCF-DA at an optimized concentration between 1-10 uM[46]. Fluorescence is monitored at Ex485/Em530. I expect that cells treated with higher concentrations of disinfectants may exhibit increased ROS production. I also predict that ROS production will correlate to the rate of HGT seen in the competence and conjugation assays, as well as cell death. Possibly there is a threshold for ROS levels and at a certain concentration the cells are "pushed over the edge" and die, but below this concentration, we see secondary HGT effects.

Examine the adaptive evolution and horizontal gene transfer ability of specific species grown constantly in sub-MIC concentrations of disinfectants

I wanted to understand the potential effects of long-term exposure to sub-inhibitory levels of disinfectants. First, do potential pathogens show adaptive resistance to long-term disinfectant exposure? Does disinfectant treatment increase resistance to that same disinfectant, other disinfectants, or antibiotics? Second, constant growth in sub-MIC concentrations of disinfectants is similar to conditions in which hospital surface bacteria might be found. Does constant sub-MIC growth increase HGT?

To evolve bacteria under controlled laboratory conditions, I start to grow each strain in its preferred media, with the addition of disinfectants at a concentration killing 50% of the bacteria as determined in the MIC experiment[47]. Each day 1% of the culture are diluted into fresh medium. Every four days the disinfectant concentration is increased by 1.5 times[47]. After five disinfectant concentration increases, the sample MICs for the disinfectants and antibiotics are measured again. Disinfectant concentration continue to be increased another five times before sampling again. The experiment continues until reaching a disinfectant MIC (original or new) for each strain. At the end of the experiment, MICs of antibiotics and disinfectants are measured again. Samples are also taken every four days and stored at -80 C for later analysis. Because this evolutionary protocol often leads to extinction of bacteria, 96 parallel cultures for each test are propagated. Lazar *et al.* successfully used this method to evolve *E. coli* under antibiotic stress for 240-384 generations, ending the experiment once 10/96 populations showed growth or the antibiotic solubility limit was reached[47]. Applying this method to the disinfectants should be attainable.

Table 3: Antibiotics to use in adaptive resistance experiments.

Antibiotic	**Antibiotic Class**
Tetracycline	Tetracyclines
Ciprofloxacin	Fluoroquinolones
Penicillin	β-lactams
Erythromycin	Macrolides
Sulfamethoxazole	Sulfonamides
Gentamycin	Aminoglycosides

It has previously been shown that triclosan, a common biocide product found in hundreds of consumer products, including toothpaste and soaps, induces multi-drug resistance in *E. coli* treated with sub-MIC levels for 30 days[48]. I expected that at the end of my adaptive evolution experiment, some bacteria would have higher MICs to the disinfectant used. I also expected cross-resistance to occur, where some bacteria have developed resistance, not only to the disinfectant, but to an antibiotic or another disinfectant acting through a similar pathway. Although more common for antibiotics of the same functional class, cross-resistance is seen in antibiotics acting mechanistically different[47]. I perform metagenomic sequencing to determine mutations contributing to adaptive resistance to the disinfectants.

To mimic long-term exposure to sub-MIC disinfectants, I grow each species for 30 days in constant disinfectant concentrations 200x, 100x, and 50x below its MIC. Controls grown without disinfectants are used. In addition, to assess HGT, I add characterized plasmids carrying resistance to the tested antibiotics (Table 3). Each day, 1% of the cultures is transferred to fresh media with fresh plasmids. After the time-course collection of samples, I perform qPCR on the samples for the spiked in ARGs. Before the qPCR, I treat with Dnase to remove any DNA in the media. Alternately, I could plate serial dilutions directly onto selective plates to assay for the presence of transformants. Sanger sequencing for each ARG could determine if the colony is a transformant or resistant due to a mutational effect of the disinfectant. I expect that constant growth in sub-MIC disinfectants will increase the rate of antibiotic resistant transformants. I also envision some

species would have been able to pick up the majority of ARGs, like *A. baylyi*, whereas some, which are less naturally competent to start, may have picked up fewer ARGs. I realize that this assay for transformants cannot distinguish between transformant daughter cells or transconjugants. But, I am more concerned with an overall increase in HGT in the disinfectant treated samples compared to the untreated controls.

Significance

Hospital-acquired infections are an increasingly prevalent issue in the United States. An estimated 99,000 deaths each year in the U.S. are a result of nosocomial infections[1]. It is estimated that the overall direct medical costs incurred from nosocomial infections is between $35.7 and $45 billion[49]. Because many of these pathogens are becoming increasingly multi-drug resistant, it is likely that the number of deaths associated with these infections will increase, unless steps are taken to mitigate the problem. I have proposed studies to understand the potential impact disinfectants have on the spread of antibiotic resistance genes. I explore the ability of contaminating DNA to be transformed and elucidate possible effects on horizontal gene transfer following disinfectant treatment. With this proposal, I hope to be able to draw conclusions that will enhance our understanding of how antibiotic resistance genes move in surface communities and inform best practices for disinfection in hospitals.

CHAPTER 3

PHAGE AND PLASMID

Because bacteriophage and plasmids are main constituents carrying antibiotic resistance genes, it is also interesting to consider the roles both play in harboring and transferring ARGs. I have developed and optimized phage and plasmid isolation from human and mouse stool protocols[50,51]. The phage isolation begins with filtering out stool particles and both eukaryotic and prokaryotic cells. Density gradient ultracentrifugation using a Cesium Chloride gradient of 1.35, 1.5, and 1.7 g/mL is then performed. Following centrifugation, phage are extracted just below the 1.5g/mL density line. From here, virions are extracted and nucleic acid amplification is performed with an isothermal phi29 polymerase. Samples are then ready to be library prepped for sequencing. Phage isolation was verified in a number of ways: 1. SYBR-gold staining of viral particles was performed; 2. Transmission Electron Microscopy (TEM) was performed (with little success due to sample preparation difficulty); and 3. CrAssphage PCR. CrAssphage is a common bacteriophage found in about 70% of humans and is the most abundant bacteriophage in publicly available metagenomes[52]. Using Primers from Dulith *et al*. I was able to confirm virion isolation from human stool[52].

The plasmid isolation protocol involves isolation of stool cells and a hot alkaline-lysis of bacteria[51]. DNA is extracted and amplified with phi29 polymerase. To verify the protocol was working, I performed plasmid spike-ins and qPCR to examine known plasmid recovery. Contamination checks were also performed with qPCR of 16s and 18s genes.

To begin to use these protocols and examine the data output, two healthy U.S. donor samples and five time-points of a neutropenic bone marrow transplant patient receiving antibiotics, were processed. The neutropenic patient comes from a cohort at Weill Cornell Medicine and a

collaboration with Dr. Michael Satlin. As neutropenic patients are more likely to be infected with an MDR pathogen, due to little immune function, these patients are a good model to investigate potential reservoirs and transfer of antibiotic resistance genes during prophylactic antibiotic treatment.

After sample preparation and sequencing of these phage and plasmid preparations, analysis followed. Reads were quality controlled with trimming, dereplication, and quality filtering.
Phage sequencing analysis began with read assembly with IDBA. The assembled reads were BLASTed against the PhAnToMe database of over 2,000 annotated bacteriophage. (note: this website/database no longer exists.) Open reading frames were called with Prodigal. Proteins and ARGS were found with Shortbred and the resfams database. RPKM was also calculated. Plasmid sequence analysis began with read assembly using plasmidSPADES. The assemblies were BLASTed against a curated set of reference plasmid genomes from NCBI. Again, Prodigal, resfams, and RPKM annotated the data.

Preliminary results suggest numerous phage detected in each sample. Phage identity varied between time-points for the neutropenic patient (Table 4). Contig annotation with number of ARGs present showed most contigs contained only one ARG, while some contigs contained up to three ARGs (Figure 3). The number of annotated plasmids for each sample was very low. No sample contained more than three annotated plasmids. This leads me to believe that this data is not reliable, as it is unlikely that there are so few plasmids and plasmid-types present in a healthy human gut. In fact, just looking at the most abundant resistance plasmids across the Human Microbiome stool dataset reveals ten different plasmids, present in up to 90% of samples[53]. Therefore, plasmid analysis was less successful.

Overall, phage and plasmid specific amplification and sequencing provide another layer of understanding to the gut microbiome and its pool of antibiotic resistance genes and mobile elements. These methods would be best suited to be combined with metagenomic sequencing and potentially another method of linking mobile elements to resistant bacteria, such as single-cell or isolate sequencing. Information from these multiple sequencing methods would provide verification and deeper information of the gut microbiome.

These protocols can be used for a number of studies. As mentioned above, monitoring of changes in mobile element and ARG pools over a time-course, can be done. This is particularly interesting to look at in these neutropenic patients who are highly susceptible to infection. This can also be applied to other time-course studies. For example, I began a mouse study looking at the effects of colitis-induced inflammation and anti-oxidant protective effects on the pool of mobile elements and ARGs in the mouse gut. These protocols are also useful when amplification of the mobile elements of the microbiome is necessary for further downstream experiments and analysis.

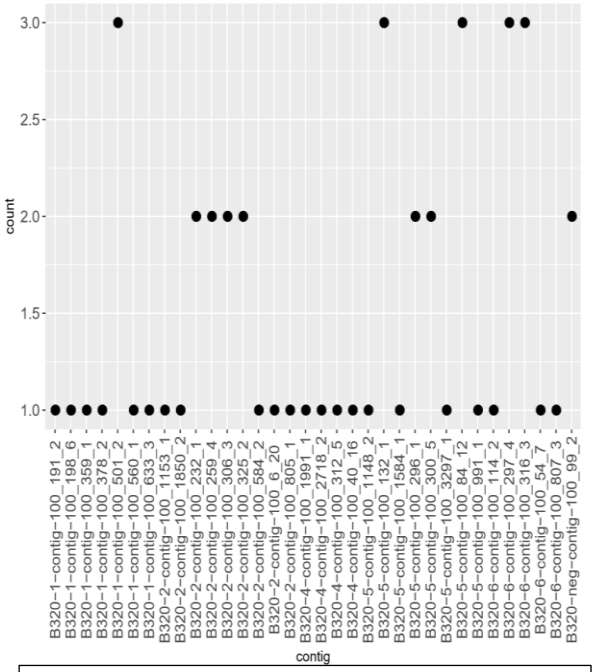

Figure 3: Number of ARGs per phage contig of neutropenic bone marrow

Table 4: Bacteriophage present for CDC neutropenic patient B320 during time-course.

TP1	TP2	TP4	TP5	TP6
Burkholderia phage KS5	Acinetobacter phage Ac42	Bacillus phage Bobb	Bacillus phage Bobb	Acinetobacter phage Ac42
Burkholderia phage ST79	Bacillus phage Bobb	Burkholderia phage KS5	Burkholderia phage Bcep176	Bacillus phage Bobb
Escherichia phage Lw1	Burkholderia phage Bcep176	Burkholderia phage ST79	Burkholderia phage KL3	Burkholderia phage KS5
Pseudomonas phage F116	Burkholderia phage KL3	Cronobacter phage CR9	Burkholderia phage KS5	Burkholderia phage ST79
Ralstonia phage phiRSA1	Burkholderia phage KS5	Enterobacteria phage GEC-3S	Burkholderia phage KS9	Chronobacter phage vB_CsaM_GAP32
Shigella phage SfII	Burkholderia phage KS9	Erwinia phage phiEaH2	Burkholderia phage ST79	Erwinia phage phiEaH2
Stx converting phage vB_EcoP_24B	Burkholderia phage ST79	Escherichia phage HK75	Cronobacter phage CR9	Pseudomonas phage F116
	Edwardsiella phage GF-2	Pseudomonas phage PaBG	Erwinia phage phiEaH2	Pseudomonas phage PaBG
	Erwinia phage phiEaH2	Ralstonia phage phiRSA1	Pseudomonas phage PaBG	Ralstonia phage phiRSA1
	Escherichia phage D108	Shigella phage SfII	Pseudomonas phage phiKZ	Shigella phage SfII
	Escherichia phage HK75		Ralstonia phage phiRSA1	Stenotrophomonas phage Smp131
	Pectobacterium phage ZF40		Shigella phage SfII	Burkholderia phage phiE125
	Pseudomonas phage D3		Burkholderia phage phiE125	
	Pseudomonas phage F116			
	Pseudomonas phage PaBG			
	Ralstonia phage phiRSA1			
	Salinivibrio phage CW02			
	Shigella phage SfII			
	Burkholderia			

CHAPTER 4

CONCLUSION

As previously mentioned, MDR pathogens are becoming increasingly common in healthcare settings and nosocomial infections cause thousands of deaths and billions of dollars lost per year. The implications of improper disinfection or unknown side-effects of surface disinfection may be large. This thesis aimed to discuss the role of disinfectants in horizontal gene transfer of antibiotic resistance genes, potentially playing a negative role in nosocomial MDR infection rate.

We need to identify the best strategies for nosocomial infection prevention and hospital disinfection is a key component needed to ensure safety from infectious diseases. It is important to understand the mechanism of disinfectants on human commensals and pathogens, if we want to be successful in containing the spread of pathogens and ARGs. There may be unintended consequences that need to be identified. Further investigation of disinfectants and horizontal gene transfer will allow better informed cleaning protocols and potentially reduce the number of MDR-nosocomial infections. Further, methods that allow discernment of mobile elements carrying ARGs, such as phage and plasmid isolation, will provide a deeper understanding of the mobile resistome pool in hospitals and microbiomes.

As antibiotic resistance increases, it is important to not only discover or synthesize new antimicrobial compounds for antibiotic use, but biocides as well. As resistance to biocides has been shown, in the future, our current disinfectants may be less effective against MDR pathogens. It is important to understand the possible mechanisms of resistance and why they occur, to make the best decisions about which product to use when and where, as well as for how long.

To best inform practice and policy, it is important to have wide knowledge of all aspects of disinfection. Each nosocomial pathogen has its own characteristics and may respond very

differently to a specific disinfectant than another pathogen. Tailoring disinfection strategies for different hospitals could be effective in reducing nosocomial infection rates. Acute care hospitals and chronic care hospitals may benefit from different disinfection strategies. If certain hospitals have higher rates of infection of specific pathogens, it is important that the disinfection strategy there, targets that pathogen. In developing countries, rates of drug-resistant *Tuberculosis* are higher than in the U.S., and disinfectants more effective against this pathogen may be necessary. Global information needs to be considered as policy and practices that work in the U.S. may need to be adjusted in other countries with different common MDR diseases.

REFERENCES

1. CDC (2014). Healthcare-associated infections. www.cdc.gov.
2. Magill *et al.* (2014) Multistate point-prevalence survey of health care-associated infections. *N Engl J Med* **370**:1198-1208.
3. CDDEP (2018) Resistance map. http://resistancemap.cddep.org.
4. Zhang S. (2017) A woman was killed by a superbug resistant to all 26 American antibiotics. *The Atlantic.*
5. Braoudaki M, Hilton AC. (2004) Adaptive resistance to biocides in *Salmonella enterica* and *Escherichia coli 0157* and cross-resistance to antimicrobial agents. *J Clin Microbiol* **42**(1):73-78.
6. Kramer A, Schwebke I, Kampf G. (2006) How long do nosocomial pathogens persist on inanimate surfaces? A systematic review. *BMC Infectious Diseases* **6**:130.
7. McDonnell G and Russell A. (1999) Antiseptcics and disinfectants: activity, action, and resistance. *Clinical Microbiology Reviews* 147-179.
8. Barbusinski K. (2009) Fenton reaction-controversy concerning the chemistry. *Ecol Chem Eng* **16**(3):347-358.
9. Rutala W, Weber D, HICPAC. (2008) Guideline for disinfection and sterilization in healthcare facilities, 2008. *CDC.*
10. Estrela *et al.* (2002). Mechanism of action of sodium hypochlorite. *Braz Dent J* **13**(2):113-117.
11. Chapman J. (2003) Biocide resistance mechanisms. *International Biodeterioration and Biodegradation* **51**:133-138.
12. Poole K. (2002) Mechanisms of bacterial biocide and antibiotic resistance. *J Appl Microbiol* **92**:Suppl55S-64S.
13. Chapman J. (2003) Disinfectant resistance mechanisms, cross-resistance, and co-resistance. *International Biodeterioration and Biodegradation* **51**(4):271-276.
14. Pal C, Bengtsson-Palme J, Kristiansson E, Larsson DGJ. (2015) Co-occurrence of resistance genes to antibiotics, biocides and metals reveals novel insights into their co-selection potential. *BMC Genomics* **16**:964.
15. Andersson D, Hughes D. (2014) Microbiological effects of sublethal levels of antibiotics. *Nature Rev Microb* **12**:465-478.
16. Kelley W. (2006) Lex marks the spot: the virulent side of SOS and a closer look at the LexA regulon. *Mol Microbiol* **62**(5):1228-38.
17. Michel B. (2005) After 30 years of study, the bacterial SOS response still surprises us. *PLoS* **3**(7):e255.
18. Charpentier X, Polard P, Claverys J-P. (2012) Induction of competence for genetic transformation by antibiotics: convergent evolution of stress responses in distant bacterial species lacking SOS? *Curr Opin Microbiol* **15**(5):570-576.
19. Erill I, Campoy S, and Barbe J. (2007) Aeons of distress: an evolutionary perspective on the bacterial SOS response. *FEMS Microbiol Rev* **31**:637-656.
20. Beaber J, Bianca H, Waldor M. (2003) SOS response promotes horizontal dissemination of antibiotic resistance genes. *Nature* **427**:72-74.
21. Ubeda C *et al.* (2005) Antibiotic-induced SOS response promotes horizontal dissemination of pathogenicity island-encoded virulence factors in staphylococci. *Mol Microbiol* **56**(3):836-844.
22. Bhattacharya G, Dey D, Das S, Banerjee A. (2017) Exposure to sub-inhibitory concentrations of gentamycin, ciprofloxacin and cefotaxime induces multidrug resistance and reactive oxygen

species generation in methicillin-sensitive *Staphylococcus aureus*. *J Med Microbiol* **66**(6):762-769.
23. Shun-Mei E *et al.* (2017) Sub-inhibitory concentrations of fluoroquinolones increase conjugation frequency. *Microbial Pathogenesis* **114**:57-62.
24. Bearson BL, Brunelle BW. (2015) Fluoroquinolone induction of phage-mediated transfer in multidrug-resistant *Salmonella*. *Int J Antimicrob Agents* **46**(2):201-204.
25. Jutkina J, Marathe NP, Flach C, Larsson DGJ. (2017) Antibiotics and common antibacterial biocides stimulate horizontal transfer of resistance at low concentrations. *Science of the total Environment* **616-617**:172-178.
26. Zhang Y, Gu AZ, Cen T, Li X, He M, Li D, Chen J. (2018) Sub-inhibitory concentrations of heavy metals facilitate the horizontal transfer of plasmid-mediated antibiotic resistance genes in water environment. *Environ Pollut* **237**:74-82.
27. Zhang Y, Gu AZ, He M, Li D, Chen J. (2017) Subinhibitory concentrations of disinfectants promote the horizontal transfer of multidrug resistance genes within and across genera. *Environ Sci Technol* **51**(1):570-580.
28. Lax *et al*., (2017) Bacterial colonization and succession in a newly opened hospital. *Science Translational Medicine* **9**(391).
29. Weinstein R. and Hota B. (2004) Contamination, disinfection, and cross-contamination: Are hospital surfaces reservoirs for nosocomial infection? *Clin Infect Diseases* **39**(8).
30. Messina G. *et al*., (2013) Environmenal Contaminants in hospital settings and progress in disinfecting techniques. *Biomed Research International* **2013.**
31. Thompson LR *et al.* (2017) A communal catalogue reveals Earth's multiscale microbial diversity. *Nature* **551**:457-463.
32. Caporaso JG *et al.* (2010) QIIME allows analysis of high-throughput community sequencing analysis. *Nature Methods* **7**:335-336.
33. Chu ND., *et al.* (2017) Profiling living bacteria informs preparation of fecal microbiota transplantations. *PLoS ONE* **12**(1): e0170922.
34. Fournier PE *et al.* (2006) Comparative genomics of multidrug resistance in *Acinetobacter baumannii*. *PLoS Genet* **2**(1):e7.
35. Poirel L, Bonnin RA, Nordmann P. (2011) Genetic basis of antibiotic resistance in pathogenic *Acinetobacter* species. *IUBMB Life* **63**(12):1061-1067.
36. Qin H. *et al*., (2018) Comparative transcriptomics of multidrug-resistant *Acinetobacter baumannii* in response to antibiotic treatment. *Scientific Reports* **8**(3515).
37. Thomas CM, Nielson KM. (2005) Mechanisms of, and barriers to, horizontal gene transfer between bacteria. *Nat rev Microbiol* **3**(9):711-721.
38. Averhoff B. and Graf I. (2008) The natural transformation system of *Acinetobacter baylyi* ADP1: A unique DNA transport machinery. *Acinetobacter Molecular Biology.* Caister Academic Press UK.
39. Elliot KT and Neidle EJ. (2011) *Acinetobacter baylyi* ADP1: Transforming the choice of model organism. *IUBMB Life* **63**(12):1075-1080.
40. Zerbino DR and Birney E. (2008) Velvet: Algorithms for de novo short read assembly using de Bruijn graphs. *Genome Research* 18:821-829.
41. Katoh, Misawa, Kuma, and Miyata. (2002) MAFFT: a novel method for rapid multiple sequence alignment based on fast Fourier transform. *Nucl Acids res* **30**:3059-3066.
42. Jia *et al.* (2017) CARD 2017: expansion and model-centric curation of the Comprehensive Antibiotic Resistance Database. *Nucleic Acids Research* **45**:D566-573.

43. Gibson MK, Forsberg KJ, Dantas G. (2014) Improved annotation of antibiotic resistance functions reveals microbial resistomes cluster by ecology. *ISME Journal* 1-10.
44. Wiegand I, Hipert K, Hancock REW. (2008) Agar and broth dilution methods to determine the minimal inhibitory concentration (MIC) of antimicrobial substances. *Nature Protocols* **3**:163-175.
45. Klumper U *et al.* (2015) Broad host range plasmids can invade an unexpectedly diverse fraction of a soil bacterial community. *ISME Journal* **9**:934-945.
46. Eruslanov E, Kusmartsev S. (2010) Identification of ROS using oxidized DCFDA and flow-cytometry. *Methods Mol Bio* **594**:57-72.
47. Lazar *et al.* (2013) Bacterial evolution of antibiotic hypersensitivity. *Mol Syst Biol* 9:700.
48. Lu *et al.* (2018) Non-antibiotic antimicrobial triclosan induces multiple resistance through genetic mutation. *bioRxiv.*
49. Scott RD. (2009) The direct medical costs of healthcare-associated infections in U.S. hospitals and the benefits of prevention. *CDC.*
50. Thurber R., *et al.* (2009). Laboratory procedures to generate viral metagenomes. *Nature Protocols* **4**: 470-483.
51. Kav AB., *et al.* (2012). Insights into the bovine rumen plasmidome. *PNAS* **109**(14): 5452-5457.
52. Dutilh B. *et al.* (2014) A highly abundant bacteriophage discovered in the unknown sequences of human faecal metagenomes. *Nature Comm.* **5**(4498).
53. Broaders E., Cormac G.M., Gahan & Julian R. Marchesi (2013) Mobile genetic elements of the human gastrointestinal tract. *Gut Microbes* **4**(4) 271-280.